REMARKS/ARGUMENTS

Claims 1-13 are pending in the present application. Claims 1, 5 and 13 are rejected and claims 2-4 and 6-12 are objected to in the present Office Action. Claim nos. 1, 5 and 13 are, thus amended herein to more clearly define the present invention. These amendments are supported by the application as originally filed and thus there is no issue of new matter raised by the amendments. Entry of this Amendment into the file of the present application is respectfully requested as it is believed to place the entire application in condition for allowance or, at a minimum, to materially reduce the issues for an appeal.

Rejections Under 35 U.S.C. 112, Second Paragraph

Claims 1 and 13 are rejected under 35 U.S.C. 112, second paragraph, for the reasons set forth on p. 2 of the Office Action. These rejections are respectfully traversed.

In response to the above rejection, the subject claims have been amended to delete the language which the Examiner indicated as being 'confusing' and to more clearly describe what applicants deem to be their invention. These amendments are believed to overcome the Examiner's grounds for rejection under §112, second paragraph and the Examiner is, thus, respectfully requested to reconsider and withdraw the rejection of claims 1 and 13.

Rejections Under 35 U.S.C. 112, First Paragraph

Claims 5 and 13 are rejected under 35 U.S.C. 112, first paragraph as set forth at pps. 2-4 of the Office Action. The Examiner alleges that the subject claims do not comply with the 'enablement requirement of 35 U.S.C. 112, first paragraph, in that they contain subject matter that was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. This ground of rejection is respectfully traversed.

In response to the rejection, applicants have amended the language of claim 5 to recite that the endonuclease according to the invention cleaves the DNA, "between the fourth and fifth bases 3'of said particular nucleotide sequence and, in the complementary strand, between the fifth and sixth bases 5' of the complement of said particular nucleotide sequence". Additionally, claim 13 is amended to now recite that the enzyme specifically recognizes DNA at a particular

nucleotide sequence and that it cleaves the DNA, "between the fourth and fifth bases 3' of said particular nucleotide sequence and, in the complementary strand, between the fifth and sixth bases 5'of the complement of said particular nucleotide sequence. As amended, the subject claims are believed to fully meet the 'enablement requirement' of section 112, first paragraph and the Examiner is, therefore, respectfully requested to reconsider and withdraw the rejection.

Notwithstanding the above, applicants note that the Examiner indicates in the Office Action that he found the arguments made in the previous response dated August 25, 2006 against the §112, first paragraph, rejection of claims 5 and 13 to be confusing. The following discussion is, therefor, intended to clarify the record with regard to the arguments made by applicants at pps. 7-10 in their August 25, 2006 Amendment.

Prior to clarifying their previous arguments, however, applicants note the Examiner's statement on p. 3 (lines 14-17) of the present Office Action that, ". . . . [t]he second, third and fourth positions in the figure on page 9 of the amendment shows cleavage as being through a base (line through the letter), not to the left or right of the base." Applicants apologize for any confusion caused to this typographical error in the preparation of the indicated figure and submit that a corrected replacement for the subject figure, wherein the cleavage lines pass between the relevant bases, is included at the end of this response. The Examiner is respectfully requested to enter the new figure as a replacement for the original figure at page 9 of applicants' prior response.

As indicated at p. 9 of the August 25, 2006 Amendment, in the present invention lambda phage DNA was digested with HpyC1I to determine the recognition and cleavage site of HpyC1I. Because the digested restriction fragment may have a sticky end, to facilitate the following ligation step, the restriction fragments are blunted by T4 DNA polymerase (see the figure on p. 10 of the August 25th response) then cloned into the EcoRV site of pBR322 plasmid. The restriction fragment-vector junction was then sequenced. Both the sequences of pBR322 plasmid and lambda phage DNA are already known, the inserted fragment will be known and the restriction fragment-vector junction will also be known. Comparing the 10 junction sequences in the original lambda phage DNA (as shown in the replacement for the figure originally provided on p. 9 of applicants' August 25th response, which replacement figure is appended at the end of this response), a recognition site (5'-CCATC-3') was identified at a constant distance from the junction. When the recognition site (5'-CCATC-3') is located in the cloned HpyC1I restriction

fragments, there will be 5 base pairs between the recognition site and the junction. On the other hand, if the recognition site (5'-CCATC-3') is not located in the cloned HpyC1I restriction fragments, there will be only 4 base pair between the recognition site and the junction. The foregoing comparison thus permitted the inventors to deduce that HpyC1I recognizes a sequence 5'-CCATC-3' and cleaves DNA between the fourth and fifth bases 3' of the particular nucleotide sequence and, in the complementary strand, between the fifth and sixth bases 5' of the complement of the particular nucleotide sequence.

Paragraph 14 of the present specification states that "a non-palindromic **recognition sequence of 5'-CCATC-3'**(designated SEQ ID NO : 1) and cleaves the fourth base **downstream from the recognition sequence** of the upper strand and the fifth base from that of the lower strand of SEQ ID NO : 1." Based on the above, the inventors determined that "downstream" as used in the specification means recognition site (5'-CCATC-3') not its inverse complement. Applicants further stated in their prior response that the arrow in the illustration on p. 9 thereof (which is to replaced by the corrected figure provided herewith) indicates the junction site, **not** the cleavage site. The Office Action indicates, however, that there is some confusion on the part of the Examiner regarding applicants' discussion of the "junction site". A further discussion concerning such 'junction site' is therefore provided herein.

The Examiner is respectfully informed that the so-called "junction site" is not a new site created along the 'string' of nucleotides. It refers, in fact, to a reference site well known among those having ordinary skill in this art for analyzing the cleavage site of restriction enzymes. The use of such "junction sites" is demonstrated, for example, in Zylicz-Stachula, A., *et al.*, "TspGWI, a thermophilic class-IIS restriction endonuclease from *Thermus sp.*, recognizes novel asymmetric sequence 5'-ACGGA($N_{11/9}$)-3'", *Nucleic Acids Research*, (2002) Vol. 30, no. 7, e33 and Skowron, P. M., *et al.*, "A new *Thermus sp.* Class-IIS enzyme sub-family: isolation of a 'twin' endonuclease TspDTI with a novel specificity 5'-ATGAA($N_{11/9}$), related to TspGWI, TaqII and Tth111II", *Nucleic Acids Research* (2003) Vol. 31, No. 14, e74, copies of which are provided with this response. As shown by these references, for purposes of analysis, a λ DNA was digested with a restriction enzyme and the restriction fragments obtained were then subcloned to backbone vectors in order to proceed with a sequence analysis. Consequently, four types of cloning construct, in which the sticky ends were blunted by polymerase, were generated, as shown in the figures provided at pps. 9-10 of applicants' previous response (with the figure

from p. 9 now being substituted by the replacement figure provided herewith) and described at p. 8 of applicants' Amendment dated August 25, 2006.

As indicated, before being cloned into the pBR322 plasmid, the restriction fragments are blunted by T4 DNA polymerase, which conclusively demonstrates that the junction site is not the cleavage site. Instead, the junction site may be off from the cleavage site by a base more or less. Thus, the base 'beside' the cleavage site serves as a junction for ligation in a subsequent cloning step. It is, in fact, due to this function that the site is referred to as a "junction site" and it is not meant to be confused with the cleavage site.

Applicants thus trust that the explanation provided above will serve to clarify that the vertical arrow shown in Table 1 on p11 refer to the junction site, instead of to the cleavage site. To avoid confusion between the junction site and the cleavage site, applicants have reconfigured the information found in Table 1 into a revised chart wherein a portion of the sequences listed at positions 1635-1596, 5009-4970, 9894-9855, 12443-12404 and 39627-39588 in Table 1 has been amended by replacement with the sequence of the strand reading from 5' to 3' such that, consequently, the junction site matches up with the cleavage site. This revision to Table 1, prepared by applicants to clarify the relation between the 'junction site' and the 'cleavage site', is provided below.

position in *lamda* DNA	*DNA sequences flanking HpyC1I cleavage sites in *lamda* DNA
1325-1364	5'-*CTGGCCAAAGTCCATCCGTG*↓**GCTCCACGCCAAAAGTGAGA**-3'
1635-1596	5'-*ACCAGAGAATGCCATCACGG*↓**GTCCAGATCCCGGTCTTTTC**-3'
4797-4836	5'-*TGCTCGATATGGACACGCCC*↓**GGCGGGATGGTGGCGGGGGC**-3'
5009-4970	5'-*AATTACTGTGAGCCATCATG*↓**ACGCCGATGGAGCCTGTCCG**-3'
9581-9620	5'-*CAGTGGTATGACCATCACCG*↓**TGAACGGCGTTGCTGCAGGC**-3'
9894-9855	5'-*TCGCCACCAGAAACGCGCCG*↓**GTTCTGATGGCGTCTTCCAC**-3'
11833-11872	5'-*TCCTGCAGGCGGATTACAAC*↓**ACGCTGATGGCGGCGGCGAA**-3'
12443-12404	5'-*CTTCAGGCCTGCCATCCAGT*↓**TCCCGCGAAGCTGGTCTTCA**-3'
39312-39351	5'-*AGACTATCGCACCATCAGCC*↓**AGAAAACCGAATTTTGCTGG**-3'
39627-39588	5'-*GTCAAAGTTAACCATCTGTG*↓**CGGCGATGTTTTTCATAGAT**-3'

Applicants trust that the comments provided above will serve to clarify for the Examiner the arguments set forth in their prior response and that the subject arguments, taken together with the claim amendments described herein, will lead to the reconsideration and withdrawal of the

'non-enablement' rejection of claims 5 and 13 under 35 U.S.C. §112, first paragraph in order that this application may proceed to issuance.

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Respectfully submitted,

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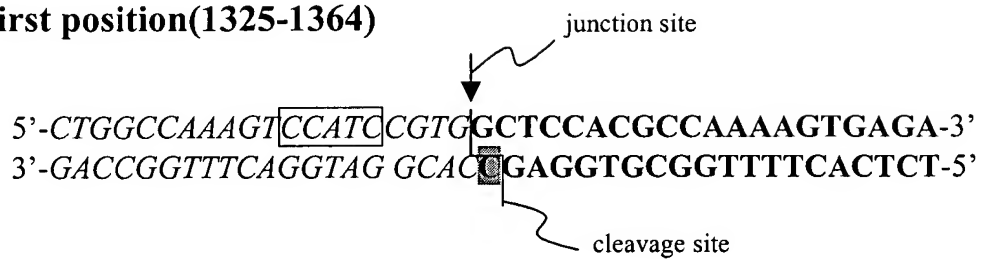
cleavage site

5'-CCATCNNNN|NNNN-3'
3'-GGTAGNNNNN|NNN-5'

first position(1325-1364)



second position(1596-1635)

5'-**GAAAAGACCGGGATCTGGA**C*CCGT*GATGG*CATTCTCTGGT*-3'
3'-**CTTTT CTGGCCCTAGACCTG** *GGCACTACCGTAAGAGACCA*-5'

third position(4797-4836)

5'-*TGCTCGATATGGACACGCCC***GGCGGGATGGTGGCGGGGGC**-3'
3'-*ACGAGCTATACCTGTGCGGG*C**CGCCCTACCACCGCCCCCG**-5'

fourth position(4970-5009)

5'-**CGGACAGGCTCCATCGGCG**T*CATGATGGCTCACAGTAATT*-3'
3'-**GCCTGTCCGAGGTAGCCGCA** *GTACTACCGAGTGTCATTAA*-5'

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A new *Thermus* sp. class-IIS enzyme sub-family: isolation of a 'twin' endonuclease TspDTI with a novel specificity 5′-ATGAA($N_{11/9}$)-3′, related to TspGWI, TaqII and Tth111II

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ABSTRACT

The TspDTI restriction endonuclease, which shows a novel recognition specificity 5′-ATGAA($N_{11/9}$)-3′, was isolated from *Thermus* sp. DT. TspDTI appears to be a 'twin' of restriction endonuclease TspGWI from *Thermus* sp. GW, as we have previously reported. TspGWI was isolated from the same location as TspDTI, it recognizes a related sequence 5′-ACGGA($N_{11/9}$)-3′ and has conserved cleavage positions. Both enzymes resemble two other class-IIS endonucleases from *Thermus* sp.: TaqII and Tth111II. N-terminal amino acid sequences of TspGWI tryptic peptides exhibit 88.9–100% similarity to the TaqII sequence. All four enzymes were purified to homogeneity; their polypeptide sizes (114.5–122 kDa) make them the largest class-IIS restriction endonucleases known to date. The existence of a *Thermus* sp. sub-family of class-IIS restriction endonucleases of a common origin is herein proposed.

INTRODUCTION

Restriction endonucleases are traditionally divided into three major classes or types: I, II and III, with the vast majority of endonucleases included in class-II (1,2).

Class-I, exemplified by EcoK and EcoB, consists of multimeric enzymes, each composed of three distinct types of subunits: S, for recognition; R, for cleavage; and M, for methylation. The endonuclease complex contains all three subunits, while the methylation complex contains only S and M subunits. The endonuclease recognizes 6–8 bp asymmetric, interrupted cognate sequence; whereas for cleavage it requires Mg^{2+}, *S*-adenosylmethionine (SAM) and ATP. SAM and ATP are utilized both as cofactors and allosteric effectors, which determine, based on methylation of substrate DNA, whether methylation or restriction activity will be turned on. During this complicated sequence of events, including ATP-driven translocation of DNA helix, scission takes place at random and far from the recognition site—up to several thousands base pairs (3,4).

Class-II was originally distinguished as featuring homodimeric or homomultimeric endonucleases, such as EcoRI, requiring Mg^{2+} as the only obligatory cofactor. They recognize 4–8 bp palindromic sequences and cleave within this sequence (1). Cognate methyltransferases are monomeric proteins, which require only SAM for activity.

In class-III, exemplified by EcoPI, an endonuclease contains two different subunits or a single subunit, and recognizes asymmetric sequences of 6 bp, cleaving outside at a distance of ~25 bp. The enzymes require Mg^{2+} as a cofactor and ATP as an allosteric activator. In addition, they are stimulated by SAM (4).

Currently, with increasing numbers of new endonucleases found—over 250 specificities and several thousands of isoschizomers (2), traditional classification is no longer adequate to cover the diversity of restriction endonucleases known. Class-II turned out to be very heterogeneous, with numerous enzymes not fitting the original classification. Several new endonuclease categories are distinguishable, either temporarily described as sub-classes within class-II or as proposed separate types. In particular, (sub)class-IIS significantly differs from the class-II paradigm by recognizing 4–7 bp asymmetric sequences, cleavage at the defined distance of 0–20 bp downstream (5), monomeric architecture (6–8) and the utilization of different mechanism of recognition and scission (9,10). Some class-IIS endonucleases, in addition to their requirement for Mg^{2+}, are stimulated by SAM, although this is not an obligatory cofactor. This unusual mode of interaction with DNA prompted detailed function–structure studies. In particular, FokI (11) has become a model protein for endonuclease–DNA interaction studies. FokI and other so far characterized class-IIS endonucleases are monomers in solution (6,12,13), although transient dimerization during cleavage has been observed (9,10). FokI and StsI both have two functional domains: one for binding to the cognate site

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and another for DNA cleavage (5,7–10,14–16). Such modular enzyme architecture allows for remarkable protein engineering experiments, such as the design and construction of artificial chimeric restriction endonucleases, composed of the FokI endonuclease cleavage domain fused with a site-specific DNA binding protein. Examples of this include *Ubx* homeodomain of *Drosophila* (17) and zinc-finger transcription factors fused to the C-terminal domain of FokI (18). Such hybrid proteins cleaved DNA at novel sites, with target recognition specificities imposed by the DNA-binding protein fusion partners. Separation of a recognition sequence from its cleavage site led to the development of a universal restriction endonuclease, capable of cleaving a single-stranded DNA target at a predetermined sequence (14,19).

Proposed class-IV is exemplified by Eco57I. The enzyme is composed of just a single polypeptide, which is a fusion of endonuclease and methyltransferase moieties. Monomeric enzyme recognizes an asymmetric sequence of 6 bp, but cleaves 16/14 bp downstream from its cognate site. It requires Mg^{2+} and is heavily dependent on SAM (20,21).

Type-BcgI-like contains unusual enzymes, which form an asymmetric protein complex of three subunits, of which two are identical. Such complexes recognize 5–7 bp continuous or interrupted asymmetric sites and can perform both cleavage and methylation. Methylation requires SAM and is stimulated by Mg^{2+}. These endonucleases cleave both upstream and downstream of the cognate site ($_{10/12}$NCGANNNNNNTGCN$_{12/10}$ in the case of BcgI), excising the recognition site along with flanking sequences from the target DNA. Cleavage requires Mg^{2+} and is stimulated by SAM (22,23).

Type-IIG partially overlaps (sub)class-IIS, class-IV and BcgI-like enzymes in that they bind to asymmetric sequences and cleave on one or both sides of their recognition sites. In addition, they are invariably stimulated by SAM, and both methyltransferase and restriction activity are located in the same polypeptide. This type is exemplified by HaeIV, which is specific to $_{7/13}$NGAYNNNNNRTCN$_{14/9}$ sequence and forms a homodimer (24), as opposed to monomeric class-IIS enzymes (6,12,13).

Type-CviJI-like contains endonucleases of eukaryotic origin, found only in viruses infecting unicellular *Chlorella* algae. The enzymes resemble class-II in their homodimeric structure and Mg^{2+} requirement as the only cofactor. However, they have features not found in any other class of restriction endonuclease: recognizing more frequent sequences than their prokaryotic counterparts—degenerated 4 bp (statistically equivalent to 3 bp), and their specificity changes in the presence of an adenine nucleotide derivative to cleave even more frequently—essentially a 2 bp sequence (25–27).

Type-BfiI-like shares characteristics of class-IIS enzymes, except it does not require Mg^{2+} for cleavage, which is a radical exception amongst restriction endonucleases of all types, and involves a different mechanism of DNA scission (28).

There are more variations within these groups or putative classes, and even evolutionary traces of other functions preceding restriction of DNA can be found, such as a ligase moiety present in NaeI. It has been shown that just a single mutation converted NaeI to topoisomerase/recombinase (29).

In this paper we present evidence for the existence of a *Thermus* sp. sub-family of related class-IIS restriction endonucleases, which have a combination of exclusive features in addition to those found in class-IIS and class-IV.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and reagents

Thermus sp. DT was from EURx Ltd bacterial strains collection, isolated during the company research program. The bacterium is an obligatory thermophile, which grows between 56 and 75°C. The optimum cultivation conditions were at 60°C in a modified Luria broth (0.5% tryptose, 0.3% yeast extract, 0.2% NaCl, pH 7.2, Nitsch's trace elements) (30). *Escherichia coli* DH11S {*mcrA* [*mrr-hsdRMS*(r_{K-}, m_K+)-*mcrBC*] Λ(*lac-proAB*) Λ(*recA1398*) *deoR*, *rpsL*, *srl*, *thi*/F′ *proAB*$^+$ *lacI*^{Q}ZΛ*M15*} (Life Technologies, Gaithersburg, MD) was used for transformation of ligation mixtures and DNA propagation.

Difco media components were from Becton-Dickinson (Franklin Lakes, NJ). Agarose GTG was from FMC (Rockland, MA). Phosphocellulose P11 resin was from Whatman (Springfield Mill, UK). Hydroxyapatite HTP was from Bio-Rad (Hercules, CA). Other chromatographic resins were from Pharmacia Biotech AB (Uppsala, Sweden). Immobilized TPCK-trypsin was from Pierce (Rockford, IL). All other reagents were from Amresco (Solon, OH) or Sigma-Aldrich (St Louis, MO), of the highest available purity.

Cloning vector pTZ18U (Ap^R, MCS, *f1 ori*, T7 promoter) (31), was obtained from Dr David Mead, Molecular Biology Inc., WI. Plasmids pBR322, pUC19 and pACYC184, mini-prep DNA purification kit, SmaI endonuclease, T4 DNA ligase, T4 DNA polymerase and lambda DNA were from EURx Ltd (Gdańsk, Poland).

TspDTI purification

The TspDTI restriction endonuclease was isolated using the following stages, performed at 4°C.

Polyethyleneimine (PEI) removal of nucleic acids. Thermus sp. DT cells were resuspended in buffer A [50 mM Tris–HCl pH 7.0, 100 mM NaCl, 5 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol (βME), 0.1% Triton X-100, 1 mM PMSF and 20 μg/ml benzamidine] and lysozyme was added to a concentration of 1 mg/ml. The suspension was incubated for 1 h and sonicated. Bacterial debris was spun down, the NaCl concentration was increased to 200 mM and PEI (pH 7.0) was added to 0.4%. The suspension was stirred for 1 h at 4°C and the nucleic acid–PEI complex was removed by centrifugation.

Ammonium sulphate (AmS) fractionation. The PEI supernatant was adjusted to 30% AmS saturation and stirred for 2 h. Precipitated contaminating proteins were removed by centrifugation and AmS was added to the supernatant to increase its concentration to 60% saturation. The protein fraction from the 30–60% AmS precipitation was spun down and the supernatant was discarded.

Phosphocellulose chromatography. Peletted TspDTI was dissolved in buffer B (20 mM KPO_4 pH 7.0, 30 mM NaCl, 0.5 mM EDTA, 5% glycerol, 5 mM βME, 1 mM PMSF, 20 μg/ml benzamidine), dialysed against buffer B and

adsorbed into phosphocellulose P11. The column was washed with buffer B and eluted with a gradient of 30 mM to 1 M NaCl in buffer B.

Heparin–agarose chromatography. Fractions containing restriction activity were dialysed against buffer C (20 mM Tris–HCl pH 7.5, 30 mM NaCl, 0.5 mM EDTA, 5% glycerol, 5 mM βME) and applied to a Heparin–agarose column. The column was washed with buffer C and TspDTI was eluted with a 30 mM to 1 M NaCl gradient in buffer C.

DEAE–Sephadex chromatography. TspDTI was dialysed against buffer D (20 mM Tris–HCl pH 7.5, 70 mM NaCl, 0.5 mM EDTA, 5% glycerol, 5 mM βME), applied to a DEAE–Sephadex column, which was eluted with buffer D.

Molecular sieving on Sephadex G-120. Active fractions from DEAE–Sephadex were concentrated to 3 ml and subjected to molecular sieving on a Sephadex G-120 column, equilibrated in buffer E [20 mM Tris–HCl pH 8.3, 3 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 25 mM KCl, 0.5 mM DTT, 5% glycerol].

Hydroxyapatite chromatography. The enzyme was further dialysed against buffer F (20 mM KPO_4 pH 7.0, 30 mM NaCl, 0.1 mM EDTA, 5% glycerol, 5 mM βME), adsorbed to a Hydroxyapatite HTP column, washed with buffer F and eluted with a 20–900 mM KPO_4 pH 7.0 gradient in buffer F. Purified, homogeneous TspDTI (Fig. 2) was dialysed against buffer G [20 mM Tris–HCl pH 7.5, 3 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 2 mM DTT, 50% glycerol] and stored at –20°C.

Determination of TspDTI recognition and cleavage sites

The TspDTI recognition site and cleavage positions were established by shotgun cloning and sequencing of the partial digestion products of bacteriophage lambda DNA. The TspDTI-generated restriction fragment ends were blunted with T4 DNA polymerase in the presence of dNTPs (30), cloned into the SmaI site of a modified pTZ18u vector (31), transformed into *E.coli* DH11S and plated onto X-Gal/IPTG plates (30). Miniprep plasmid DNA was isolated from white colonies and the fragment–vector junctions were sequenced using the ABI Prism 310 automated sequencer with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA). The obtained sequence data were then analysed using ABI Chromas 1.45 software (Perkin Elmer Applied Biosystems) and DNASIS 2.5 software (Hitachi Software, San Bruno, CA).

Proteolysis of TspGWI and amino acid sequence determination

Purified TspGWI was subjected to limited TPCK-trypsin digestion to obtain internal polypeptides for N-terminal amino acid sequencing. Proteolysis of 30 µg TspGWI was conducted in 110 µl of buffer T (20 mM Tris–HCl pH 8.3, 25 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 0.05% Tween 20, 0.5 mM DTT) with 30 µl gel-immobilized TPCK-trypsin and gentle shaking at 24°C for 3 h. The immobilized TPCK-trypsin was removed by centrifugation. The supernatant, containing TspGWI fragments was run on a 6% SDS/PAGE denaturing gel and electroblotted onto PVDF membrane in 100 mM CAPS-NaOH buffer pH 11.0. The N-terminal amino acid sequence analysis of blotted polypeptides was performed on a gas-phase sequencer (Model 491, Perkin Elmer-Applied Biosystems). The phenylthiohydantoin derivatives were analysed by online gradient high performance liquid chromatography on Microgradient Delivery System Model 140C equipped with Programmable Absorbance Detector Model 785A and Procise software (Perkin Elmer-Applied Biosystems).

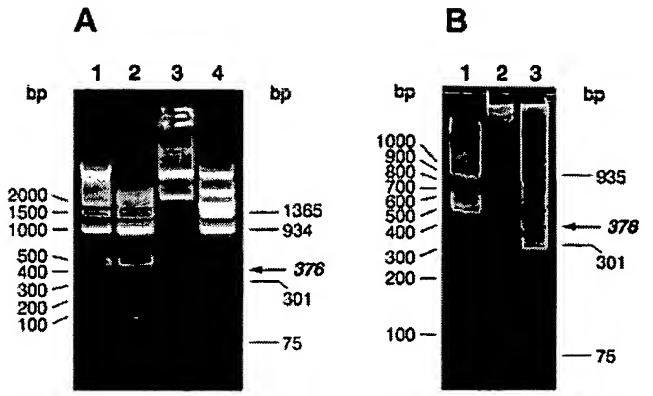

Figure 1. Partial digestion of pUC19 plasmid DNA with TspDTI restriction endonuclease. (**A**) TspDTI cleavage of pUC19 DNA, 1.5% agarose/TAE. Lane 1, 1 kb ladder; lane 2, 100 bp ladder; lane 3, untreated pUC19 DNA; lane 4, TspDTI-cut pUC19 DNA. (**B**) TspDTI cleavage of pUC19 DNA, 6% polyacrylamide/TBE. Lane 1, 100 bp ladder; lane 2, untreated pUC19 DNA; lane 3, TspDTI-cut pUC19 DNA. The smallest partial digestion band of 376 bp is indicated in bold italics with horizontal arrow.

RESULTS AND DISCUSSION

Purification and properties of *Thermus* class-IIS endonucleases

TspDTI activity is present in very small quantities in its natural host *Thermus* DT strain, thus the development of an extensive isolation procedure was essential. Seven purification stages were needed to obtain a homogeneous protein: PEI and AmS fractionations, followed by five chromatographic steps.

The optimum reaction conditions for TspDTI are in 10 mM Tris–HCl pH 8.0 at 25°C, 10 mM $MgCl_2$, 10 mM DTT. The temperature activity range extends from 42 to 85°C, with maximum activity observed at 65–75°C. Under all digestion conditions tested, a stable partial cleavage pattern was observed (Fig. 1). Spermidine does not affect TspDTI activity, while SAM stimulates the enzyme several-fold. In the presence of SAM and without Mg^{2+} the enzyme methylates its recognition site, which becomes resistant to TspDTI cleavage upon the subsequent addition of Mg^{2+} (data not shown). TspDTI can be inactivated by 20 min incubation at 89°C.

Determination of TspDTI recognition and cleavage sites

The TspDTI cleavage pattern of pUC19, pBR322, pACYC184 and lambda DNA indicated a high frequency of cleavage. The digested plasmid DNAs were run on an agarose gel (Fig. 1) and compared with the digestion patterns of known restriction endonucleases. The comparison suggested that TspDTI is an enzyme with a novel specificity. However, even repeated cleavage with concentrated TspDTI preparations failed to

Table 1. Determination of the TspDTI recognition sequence and cleavage positions by shotgun cloning and sequencing of TspDTI restriction fragments

Position in lambda genome	DNA sequences flanking *Tsp*DTI cleavage sites in bacteriophage lambda DNA. Bold, non-italics – a terminal portion of *Tsp*DTI-cut restriction fragment cloned into pTZ18u derivative; Regular, italics – not cloned bacteriophage lambda DNA sequence adjacent to cloned *Tsp*DTI-derived restriction fragment
26-61	5'-...*TATTATGAAAATTTTCCGGTTTAAGGCGTTTCCGT*...-3'
715-682*	5'-...*TTCACTCAGCAACCCCGGTATCAGTTCATCCAGC*...-3'
7207-7242	5'-...*GAAAGATGAACTGATTGCCCGTCTCCGCTCGCTGGG*...-3'
14406-14439	5'-...*GGTGGCTGGTCTGCCGGGGGACGATTCATAAGTT*...-3'
15044-15009*	5'-...*ACAATATGAATTACAGCGCCATCAGGCAGAGTCTCA*...-3'
19775-19810	5'-...*TCCGGATGAAGCCGGGCGTTACAGCATGGATGTGGA*...-3'
19910-19877*	5'-...*ATTCAGCGTCCCCGGTTGTGAATCTTCATACACG*...-3'
22821-22856	5'-...*TTTCATGAAATACATTTTTGATTATTATTTGAATC*...-3'
22952-22987	5'-...*TCCAAATGAAGCCATAGGCATTTGTTATTTTGGCTC*...-3'
23574-23539*	5'-...*TACAAATGAAAGTATGTTAAACATTGGTATAAAAAA*...-3'
24762-24797	5'-...*AAATGATGAAGAGCTCTGTGTTTGTCTTCCTGCCTC*...-3'
25085-25120	5'-...*ACCTGATGAAACAAGCATGTCATCGTAATATGTTCT*...-3'
25333-25300*	5'-...*ATGCAGATAAATGTTAGAAATAATTTCATTACTC*...-3'
26326-26291*	5'-...*AAACAATGAATATTTTTTCTCTGAAAATAATAGACT*...-3'
26515-26482*	5'-...*GCCAGAATTGTCAGATTTCCACTTTTCATTTAAT*...-3'

Table 1. *Continued*

26605-26570*	5'-...*TGAAAATGAAAGCGTCCTTAACACCTCATTACTTAG*...-3'
33084-33117	5'-...*AGACGATCCTGAATGTAATAAGCGTTCATGGCTG*...-3'
33277-33242*	5'-...*TGGCGATGAAAAGATGTTTCGTGAAGCCGTCGACGC*...-3'
35664-35699	5'-...*TTTATATGAATTTATTTTTTGCAGGGGGGCATTGTT*...-3'
42678-42711	5'-...*CAAGCCCAACAAGCCGTAAACGCGTTCATCAGAG*...-3'
42845-42812*	5'-...*CACGCATTGCTTGTGAATATTGCGTTCATTAAAT*...-3'
43176-43209	5'-...*CCATCGTCAACGACGTTCTCATGGTTCATCGCGG*...-3'
43336-43303*	5'-...*TCGAATCCAATCGTATCCAGTTTGTTCATCAGGT*...-3'
44616-44651	5'-...*GTGAGATGAAAAGAGGCGGCGCTTACTACCGATTCC*...-3'
44951-44984	5'-...*GCCGTAGCCACTGTCTGTCCTGAATTCATTAGTA*...-3'
44984-44949*	5'-...*TACTAATGAATTCAGGACAGACAGTGGCTACGGCTC*...-3'
45214-45181*	5'-...*AACAGGTCATGTTTTTCTGGCATGTTCATGTCTT*...-3'
46867-46902	5'-...*GTGCGATGAATCGTCATTGTATTCCCGGATTAACTA*...-3'
47135-47100*	5'-...*TATCCATGAACATAAAAGATATTACTATACCTTTGA*...-3'

*Sequence obtained from TspDTI restriction fragments cloned in reverse complement orientation. Base numbering refer to the conventional orientation of lambda genome; bold text, a terminal portion of TspDTI-cut restriction fragment, T4 DNA polymerase repaired and cloned into pTZ18u derivative; italic text, not cloned bacteriophage lambda DNA sequence adjacent to cloned TspDTI-derived restriction fragment; box + horizontal arrow, TspDTI recognition sequence; vertical arrows, TspDTI cleavage positions.

yield a complete reaction, resulting in a stable partial cleavage pattern, with ~50% of the substrate DNA converted to complete digestion bands (Fig. 1). Thus, the inability to completely cut substrate DNA is either an intrinsic feature of the enzyme or a key cofactor was missing in the reaction.

Comparison of the 35 junction sequences (29 are shown in Table 1) indicated that TspDTI belongs to the class-IIS restriction endonucleases, since a putative non-palindromic recognition site was found in the cloned inserts at a constant distance from the vector junction. TspDTI is a novel prototype of restriction specificity. The enzyme recognizes a 5 bp asymmetric cognate site (boxed) and cleaves DNA downstream, after nt 11 and 9 (vertical arrows) in the top and the bottom strand, respectively, yielding 2 nt 3′ single-stranded termini:

```
                                  ↓
5′-[A T G A A]N N N N N N N N N N N11 N N N-3′
3′-[T A C T T]N N N N N N N N N9  N N N N N-5′
                                  ↑
```

A computer prediction of the cleavage frequency shows that there are four TspDTI sites within pUC19, 10 within pBR322, 11 within pACYC184 and 176 within lambda DNA. However, the actual TspDTI digestion pattern exhibits more bands than expected, due to partial cleavage (Fig. 1).

Evolutionary implications

Both the recognition sequence of TspDTI and its cleavage positions appear to be related to those reported by us previously for the *Thermus* sp. restriction endonuclease TspGWI, 5′- ACGGA($N_{11/9}$)-3′ (32). The TspDTI cognate site has only two differences as compared to the 5 bp recognition site of TspGWI: a transition from C to T and from G to A in the second and fourth bases, respectively. Moreover, the cleavage positions at nt 11 and 9 are exactly the same for both enzymes. Since both TspDTI and TspGWI originate from two *Thermus* sp. isolates found in the same hot spring sample, it is possible that both enzymes occurred as a result of a recent divergent evolution event. In addition, TspDTI and TspGWI might be closely related to two previously reported class-IIS endonucleases found in different *Thermus* species: TaqII from *Thermus aquaticus* (33) and Tth111II from *Thermus thermophilus* (34), and more distantly related to mesophilic endonucleases: EciI from *E.coli* (REBASE: rebase.neb.com) and BceAI and BcefI, both from *Bacillus cereus* (35) (REBASE: rebase.neb.com) (Table 2). Both TaqII and Tth111II recognize asymmetric 6 bp redundant sites and

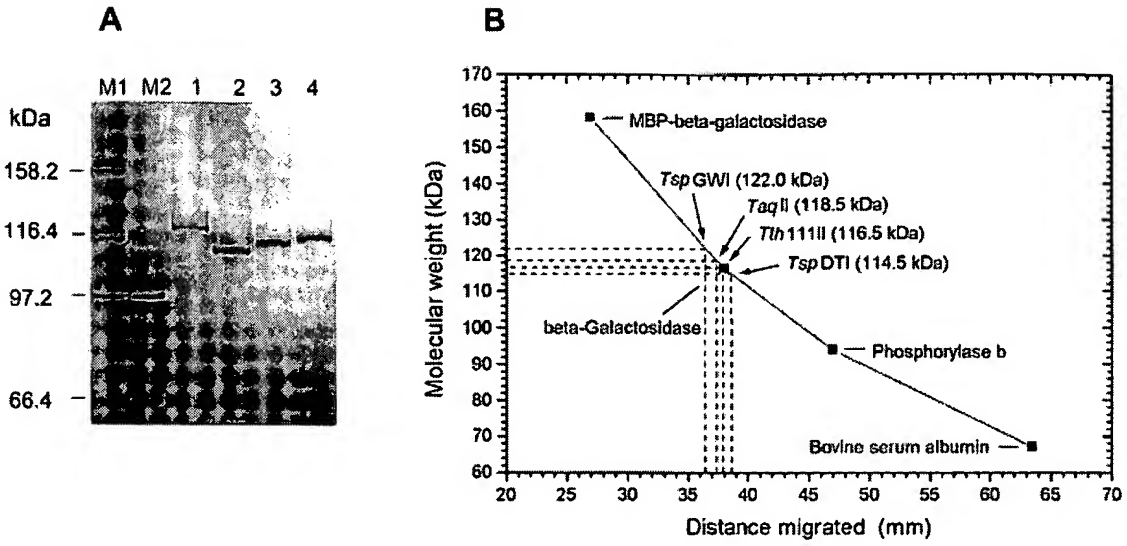

Figure 2. Determination of polypeptide molecular sizes for *Thermus* class-IIS endonucleases: TspDTI, TspGWI, TaqII and Tth111II. (**A**) SDS/PAGE of purified, homogeneous TspDTI, TspGWI, TaqII and Tth111II endonucleases. Lane M1, protein marker broad range (New England Biolabs); lane M2, low molecular weight marker (Amersham-Pharmacia). Bands marked in lanes M1 and M2 are as follows: 158.2 kDa, MBP-β-galactosidase; 116.4 kDa, β-galactosidase; 97.2 kDa, phosphorylase b; 66.4 kDa, bovine serum albumin. Lane 1, TspGWI endonuclease; lane 2, *Tsp*DTI endonuclease; lane 3, Tth111II endonuclease; lane 4, TaqII endonuclease. (**B**) Graph showing estimation of polypeptide sizes for TspDTI, TspGWI, TaqII and Tth111II.

cleave 11 and 9 nt downstream: 5′-GACCGA($N_{11/9}$)-3′ or 5′-CACCCA($N_{11/9}$)-3′ (33) and 5′-CAARCA($N_{11/9}$)-3′ (34), respectively. Due to a redundancy of TaqII and Tth111II 6 bp recognition sites, their overall cleavage frequency is only slightly lower than that of TspDTI and TspGWI 5 bp non-redundant sites. One of several possible evolutionary scenarios would be that TspGWI and TspDTI have eliminated the first C or G from an ancestral TaqII/Tth111II-like recognition sequence, thus evolving toward more frequent restriction. As illustrated in Table 2, TspGWI and TspDTI 5 bp cognate sites show 2–3 differences, when compared to those of TaqII and Tth111II. All these changes are located within a variable 3 bp 5′-TGA-3′ 'core' region (bases 2–4) of the TspDTI recognition site 5′ATGAA-3′. The first and the last A residues (bases 1 and 5) of the TspGWI and TspDTI recognition sites remain conserved amongst all four enzymes. In addition, one of the two possible variants of Tth111II cognate sites, 5′-CAAGCA-3′, also shares an internal G residue (base 4) with the TspDTI site (Table 2). Recognition sequence similarities are further validated by strict conservation of cleavage positions at nt 11 and 9 for TspGWI, TspDTI, TaqII and Tth111II. The proposal for the existence of a class-IIS sub-family, within *Thermus* sp., is further reinforced by the following findings.

(i) All the four putative related endonucleases have been purified to homogeneity from their native *Thermus* strains (A.Żylicz-Stachula, I.Sobolewski and P.M.Skowron, manuscript in preparation; S.M.Rutkowska, I.Jaworowska, I.Sobolewski and P.M.Skowron, manuscript in preparation). Strikingly, their respective polypeptides migrate to the same position on SDS/PAGE gels. Only prolonged electrophoresis allows for distinguishing subtle variations in their molecular sizes (kDa): TspDTI, 114.5 ± 7; TspGWI, 122.0 ± 7; TaqII, 118.5 ± 7; and Tth111II, 116.5 ± 7 (Fig. 2). Such large polypeptides are rare amongst prokaryotes, and to our knowledge they are the largest class-IIS restriction endonucleases known to date.

(ii) TspDTI-containing fractions eluted from the Sephadex G-120 column, used for the enzyme purification, show a homogeneous protein band, with a relative molecular weight of ~120 ± 10 kDa on SDS/PAGE denaturing gels. Relative band intensities in consecutive column fractions correlate perfectly with the restriction activity peak of TspDTI (data not shown). Subsequent comparison of the elution profiles of TspDTI, TspGWI, TaqII and Tth111II from a molecular sieving column shows that all the peaks of activity appear at nearly identical positions, characteristic of large proteins of native molecular size of 110–130 kDa (Table 2) (S.M. Rutkowska, I.Jaworowska, I.Sobolewski and P.M.Skowron, manuscript in preparation). This indicates the same, monomeric structure for TspDTI, TspGWI, TaqII and Tth111II.

(iii) TaqII restriction endonuclease has been cloned, expressed and purified in our laboratory (36). The recombinant TaqII exhibits the same molecular size as the native TaqII, which matches sequencing/genetic analysis data obtained for the *taqIIR* gene: 3315 bp/1105 aa/125.6 kDa (36; S.M. Rutkowska, I.Jaworowska, I.Sobolewski and P.M.Skowron, manuscript in preparation).

(iv) TspGWI has been subjected to partial trypsin digestion and the N-terminal amino acid sequences for two internal peptides have been determined. Comparison between two TspGWI tryptic fragments and the complete TaqII endonuclease amino acid sequence revealed near perfect homology: in peptide 1, an 8 aa continuous region contains seven identical amino acids and a single conservative substitution (100% similarity), while in peptide 2, a 9 aa region contains eight identical amino acids (88.9% similarity) (Fig. 3) (36; S.M.Rutkowska, I.Jaworowska, I.Sobolewski and P.M.Skowron, manuscript in preparation).

(v) Both TspDTI and the TaqII are capable of specific methylation of their recognition sites in the presence of SAM (Table 2) (A.Żylicz-Stachula, I.Sobolewski and P.M.Skowron, manuscript in preparation). Some data suggest that TspGWI

Table 2. Comparison between TspDTI/TspGWI endonuclease 'twins' and class-IIS restriction endonucleases with related recognition and cleavage sites

Restriction endonuclease[a]	Bacterial host[a]	Recognition site[a]	Cleavage positions[a]	Reaction temperature[a]	Polypeptide size[b]	Native molecular size[c]	Specific DNA methylation	Reference
TspDTI	***Thermus* sp.**	**ATGAA**	**$N_{11/9}$**	**70°C**	114.5 kDa	110–130 kDa	+++	This work
TspGWI	***Thermus* sp.**	**ACGGA**	**$N_{11/9}$**	**70°C**	122.0 kDa	110–130 kDa	+/–	(32), This work
TaqII	***Thermus aquaticus***	**GACCGA**	**$N_{11/9}$**	**70°C**	118.5 kDa (125.6 kDa[d])	110–130 kDa	++++	(33), (36), This work
		CACCCA	**$N_{11/9}$**					
Tth111II	***Thermus thermophilus***	**CAARCA**	**$N_{11/9}$**	**70°C**	116.5 kDa	110–130 kDa	ND	(34), This work
EciI	*Escherichia coli*	GGCGGA	$N_{11/9}$	37°C	ND	ND	ND	REBASE
BcefI	*Bacillus cereus*	ACGGC	$N_{12/13}$	30°C	ND	ND	ND	(35)
BceAI	*Bacillus cereus*	ACGGC	$N_{12/14}$	30°C	ND	ND	ND	REBASE

[a]*Thermus* sp.-derived and TspDTI/TspGWI-related endonucleases, bases in a recognition sequence, cleavage positions and reaction temperatures are marked in bold.
[b]As estimated by SDS/PAGE of homogeneous proteins.
[c]As estimated by molecular sieving under native buffer conditions.
[d]As calculated from sequencing/genetic analysis data obtained for the *taqIIR* gene.
ND, not determined.

has residual methylation activity (data not shown). Analysis of the cloned *taqIIR* gene (36; S.M.Rutkowska, I.Jaworowska, I.Sobolewski and P.M.Skowron, manuscript in preparation) reveals that it is a fusion containing both an endonuclease moiety and a methyltransferase module with FGG and DPPY motifs (37).

Taken together, the near identity of very atypical molecular sizes, substantial similarities in recognition sequences, identity in cleavage sites, strong partial amino acid sequence homology between TspGWI and TaqII, cleavage stimulation by SAM, the presence of all four endonucleases in the same bacterial genus as well as biochemical similarities point to the common evolutionary origin of TspDTI, TspGWI, TaqII and Tth111II, thus defining a sub-family of *Thermus* class-IIS enzymes. These endonucleases are characterized by a unique combination of features found only in the *Thermus* class-IIS sub-family (such as extremely large polypeptides), as well as those present in class-IIS (asymmetric cognate sequence, cleavage outside recognition site) and sub-class IV (SAM stimulation, endonuclease–methyltransferase genes fusion). Moreover, considering SAM dependence, the *Thermus* class-IIS sub-family enzymes show continuity between class-IIS and class-IV features from barely detectable (TspGWI) to strong stimulation (TaqII) (data not shown).

Three more class-IIS endonucleases from unrelated bacterial species exhibit marked similarities to the putative *Thermus* sp. class-IIS endonuclease family. The mesophilic EciI endonuclease, from *E.coli*, recognizes 5′-GGCGGA($N_{11/9}$)-3′ (REBASE: rebase.neb.com). The enzyme shares the last 4 bp out of the 5 bp of the TspGWI recognition site (bases 2–5) and has the same cleavage positions of $N_{11/9}$. Moreover, BcefI and BceAI, mesophilic endonucleases isolated from *B.cereus*, both recognize the 5′-ACGGC-3′ cognate site. Their recognition sequence differs in only one bp (last base, 5) from the TspGWI recognition sequence. However, BcefI and BceAI have cleavage positions shifted further downstream: 1 nt in the top strand and 4 or 5 nt in the bottom strand to $N_{12/13}$ or $N_{12/14}$, respectively (35; REBASE: rebase.neb.com).

In general, homologies between restriction endonucleases are very rare. They are usually limited to the epitopes, such as the highly variable catalytic motif $PDX_{10-30}(D/E)XK$ (38,39). Amongst class-IIS endonucleases, both primary sequence and

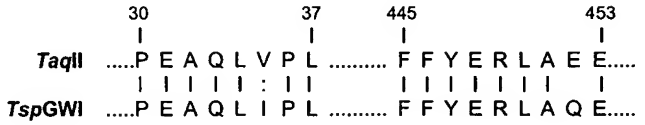

Figure 3. Amino acid sequence comparison between two tryptic fragments of TspGWI endonuclease and complete TaqII endonuclease sequence. Identical amino acid residues are indicated by straight lines, and similar residues by dots. The partial amino acid sequence of TspGWI restriction endonuclease was obtained by limited trypsin digestion, followed by N-terminal protein sequencing of two internal peptides. The TaqII amino acid sequence was translated from the cloned *taqIIR* coding gene (36; S.M.Rutkowska, I.Jaworowska, I.Sobolewski and P.M.Skowron, manuscript in preparation). The first homologous region extends from amino acid 30 to 37 and the second region from amino acid 445 to 453 of TaqII restriction endonuclease.

structural homology were reported and studied in detail for imperfect isoschizomers FokI and StsI (15,16). Both enzymes bind to the 5 bp asymmetric site 5′-GGATG-3′ and cleave 9/13 (FokI) or 10/14 (StsI) nt downstream. Remarkably, even though the enzymes exhibit relatively high amino acid sequence homology (30%), they are very distinct biochemically: StsI is an acidic protein (pI 6.3), while FokI is very basic (pI 9.4), they do not cross-react immunologically and they have different reaction optima. Nevertheless, they share a common domain organization, suggesting very close similarities in their mechanism of action (5,7–10,15–18). In contrast, two other neoschizomers belonging to class-II, SmaI and XmaI, show no homology between their amino acid sequences. Both recognize 5′-CCCGGG-3′ sites, however, they leave blunt or 4 nt sticky ends, respectively. Apparently, the mechanism of recognition is different, as they bend the DNA helix in opposite orientations (40). Very few other cases of known homologous endonucleases in class-II are limited to isoschizomers, they are listed below.

(i) EcoRI/RsrI/MunI share 18–50% homologous amino acids and common active site architecture with XcyI and Cfr9I (39,41); (ii) XmaI/XcyI/Cfr9I are highly homologous, exhibiting 80% homology between Cfr9I and XmaI/XcyI (41); (iii) AvaI/BsoBI pair, the AvaI from cyanobacteria *Anabaena variabilis* has a thermophilic counterpart from a distant species, *Bacillus stearothermophilus*. Nevertheless, the enzymes show 55% homology and possess common amino acid residues critical for catalytic activities (42,43); (iv) BsuRI

from prokaryotic *Bacillus sphaericus* and CviJI from IL-3A virus-infected eukaryotic *Chlorella*, in spite of the fact that they originate from two separate kingdoms, still exhibit 11.6% homology. Moreover, they show a substantial epitopic similarity (35%) over the 132 amino acids region (25–27,44); (v) TaqI isochizomers group contains eight related isoschizomers, with homology ranging from 54 to 100%, which was correlated with the geographical location of their *Thermus* host strains (45). Moreover, differences in their amino acid sequences allowed for an insight into observed varied thermostability (45,46). The corresponding methyltransferases are remarkably similar, indicating that both the endonuclease and the methyltransferase components of TaqI-related restriction–modification systems (RMs) were evolving as linked genes, in contrast to EcoRI and RsrI systems (47); (vi) BsuFI and MspI share 45% overall amino acid sequence similarity, including three smaller regions with 60% identity. Interestingly, in spite of their close enzyme relatedness, the *mspIRM* genes have a divergent arrangement, while *bsuFIRM* genes have convergent organization (48); (vii) EcoHK31I and EaeI share 92% identity; their corresponding methyltranferases are both composed of two homologous subunits, α and β, regulated by the same alternative open reading frame mechanism. Moreover, some evidence shows that EcoHK31I and EaeI RMs were subjected to intergenic tranfer (49); (viii) BsuBI and PstI share 46% amino acid identity. The RMs have different genetic organization: *pstIRM* genes are transcribed divergently, while *bsuBIRM* are arranged in head-to-tail orientation, with *bsuBIM* preceding *bsuBIR* (50).

According to the above examples, both primary sequences of endonuclease and methyltransferase coding genes, within related RMs, as well as the gene organization, are subjected to intense evolutionary pressure. This results in a high evolutionary rate and variability, even amongst related RMs, where methyltransferase can evolve either separately or together with an endonuclease component, or even be horizontally transferred amongst different bacterial species (49).

Whether a homology of recognition and cleavage sites of TspDTI, TspGWI, TaqII and Tth111II (possibly including EciI, BcefI and BceAI as well) is mirrored by the similarity of biochemical properties, homology and organization of their coding genes and amino acid sequences, remains to be further evaluated.

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*Tsp*GWI, a thermophilic class-IIS restriction endonuclease from *Thermus* sp., recognizes novel asymmetric sequence 5′-ACGGA($N_{11/9}$)-3′

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ABSTRACT

A novel prototype class-IIS restriction endonuclease, *Tsp* GWI, was isolated from the thermophilic bacterium *Thermus* sp. GW. The recognition sequence and cleavage positions have been established: *Tsp*GWI recognizes the non-palindromic 5-bp sequence 5′-ACGGA-3′ and cleaves the DNA 11 and 9 nt downstream in the top and bottom strand, respectively. In addition, an accompanying endonuclease, *Tsp*GWII, an isoschizomer of *Pst*I, was found in *Thermus* sp. GW cells.

RESULTS AND DISCUSSION

The general characteristics of type II restriction endonucleases are that they require Mg^{2+} ions as the only obligatory co-factor and recognize a palindromic 4–8-bp cognate site, where cleavage takes place at precisely defined positions within the recognition site (1). The type II enzymes are higly heterogeneous, with a distinct subclass, IIS, which differs from the class-II paradigm by its ability to recognize asymmetric sequences and cleave further downstream at defined distances of 1–20 nt (2). Currently there are 73 known prototypes of endonucleases that recognize asymmetric sites (1). However, only 43 of them meet the criteria of class-IIS. The remaining 30 enzymes either (i) cleave within asymmetric sites, (ii) have not yet had their cleavage positions determined or (iii) cleave on both sides outside their recognition sites (1).

Thermus sp. GW was cultivated aerobically at 60°C in a modified Luria broth (0.5% tryptose, 0.3% yeast extract, 0.2% NaCl, pH 7.2) supplemented with trace elements. Since class-IIS restriction endonuclease *Tsp*GWI activity is only present in minute quantities in the host strain, an extensive purification was required to obtain a sufficient amount of enzyme for further analysis. The purification procedure included (i) 0.4% polyethyleneimine removal of nucleic acids from the crude extract in the presence of 100 mM NaCl, (ii) ammonium sulfate 30–50% saturation cut, (iii) phosphocellulose chromatography, (iv) heparin–agarose chromatography, (v) Cibacron blue–agarose chromatography and (vi) DEAE–Sephadex chromatography. The purified preparation was free from non-specific nucleolytic activities. In the course of the purification of *Tsp*GWI an accompanying restriction endonuclease, *Tsp*GWII, a thermophilic isoschizomer of *Pst*I, was found. *Tsp*GWII recognizes 5′-CTGCAG-3′ and cleaves after the A residue, leaving 4 nt 3′-protruding ends (not shown).

The *Tsp*GWI recognition site was established using two procedures: (i) assessment of the digestion pattern on pUC19, pACYC184 and pBR322 DNAs and (ii) cloning and sequencing of *Tsp*GWI restriction fragments of pBR322 and bacteriophage lambda DNAs (Table 1). The analysis of the cleavage pattern and the mapping of *Tsp*GWI sites present in pUC19 (Fig. 1A and B) suggested that this recognition site is 5′-ACGGA-3′. There are two such sites in pUC19, four in pACYC184 and five in pBR322. Cleavage of pUC19 with *Tsp*GWI resulted in a complete digestion. However, cleavage of pBR322 and pACYC184 DNAs showed a stable partial digestion pattern, where all sites except one are efficiently cleaved (Fig. 1C–E). The refractory sites, cleaved at a substantially slower rate, were mapped and they turned out to be located within the tetracycline resistance gene, present in both plasmids. One of the possible explanations for the phenomenon of refractory cleavage might be that it is an effect of the immediate sequence segment surrounding the *Tsp*GWI site. The neighboring bases, which are present on both sides of the *Tsp*GWI recognition sequences in pBR322 and pACYC184, are shown in bold: 5′-AACGGAT-3′. The putative *Tsp*GWI cognate site was further investigated through the digestion of pBR322 DNA and lambda DNA with *Tsp*GWI, followed by repair of the *Tsp*GWI restriction fragment termini with T4 DNA polymerase/dNTPs (3) and by cloning the resulting restriction fragments into the *Sma*I site of a modified pTZ18u vector (4). The vector-insert junctions of the resulting clones were sequenced using the ABI Prism 310 automated sequencer with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit and then analyzed using ABI Sequencing Analysis 3.4.1 software (Applied Biosystems, Foster City, CA) and Hitachi DNASIS 2.5 software (Hitachi Software Engineering Co., Yokohamie, Japan). A total of 58 junction sequences were compared. Twenty-five junctions derived from bacteriophage lambda cloned restriction fragments are exemplified in Table 1, where eight junctions are for the top strand of the 5′-ACGGA-3′ and 17 for the bottom strand of the 5′-TCCGT-3′ reverse complement

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Table 1. Determination of the *Tsp*GWI recognition sequence and cleavage positions by shot-gun cloning and sequencing of *Tsp*GWI restriction fragments

Sequence position in lambda genome.	DNA sequence flanking *Tsp*GWI cleavage site in bacteriophage lambda DNA.
152-185	5'- ...*TGTCGTTTCCTTTCT*CTGTTTTTG[TCCGT]GGAAT....-3'
1064-1097	5'-...*CGTTGAGCCGACTAT*TCGTGATAT[TCCGT]CGCTG...-3'
2189-2224	5'-...*CGTCT*[ACGGA]*AAGCCGGTGGC*CAGCATGCCACGTAA...-3'
2189-2222 *	5'-...*ACGTGGCATGCTGGC*CACCGGCTT[TCCGT]AGACG...-3'
3869-3904	5'-...*AGGAT*[ACGGA]*TAACGGCTACT*CCGTGTTTGAGCAGT...-3'
4292-4325	5'- ... *ATCAGGAAATTTTTG*CCCAGCAGG[TCCGT]GAAAC...-3'
4615-4650	5'-...*TGATG*[ACGGA]*CCACGACAGGC*CCGCAGTTATCAGGT...-3'
10779-10814 *	5'-...*CAGCC*[ACGGA]*CTTTGCCCGCCT*GCAAGCTGCGTGGC...-3'
10781-10814	5'-... *CACGCAGCTTGCAGG*CGGGCAAAG[TCCGT]GGCTG...-3'
12562-12597*	5'-...*ACAGC*[ACGGA]*ACGGGTGAAGC*TGCGCCAGTTCTGCT...-3'
16454-16489	5'- ...*GCGGC*[ACGGA]*GCCGCGCATCA*CCTGTAATGCGTACC...-3'
17205-17240 *	5'- ...*TTCTC*[ACGGA]*TACTCACGCAG*CGGAACAGTCGCTGG...-3'
19472-19505	5'-...*GGTATACAGATTAAT*CCGGCAGCG[TCCGT]CGTTG...-3'
19546-19579 *	5'-...*TTTATAACCGACCCC*AACGATGAA[TCCGT]CAGTA...-3'
19924-19958	5'-...*CCATG*[ACGGA]*GGATGATGCCC*GGCCGGAGGTGCTG...3'
19977-20010	5'-...*GTGGAAGAGGTGGCG*CGTAACGCG[TCCGT]GGTGG...-3'
19975-20010 *	5'- ...*CCACC*[ACGGA]*CGCGTTACGCG*CCACCTCTTCCACCA...-3'
21994-22029	5'-...*CAACC*[ACGGA]*CCATAAAAAT*TATAATCTGCTGGCC...-3'
22319-22352 *	5'-...*CGGATCCGGAACAGT*TTTTCTGCT[TCCGT]ATCCT...-3'
30971-31004	5'-...*AAGCCCGCTGCCAGA*AAAATGCAT[TCCGT]GGTTG...-3'
31291-31324 *	5'-...*GTAGGCGCAATCACT*TTCGTCTAC[TCCGT]TACAA...-3'
32664-32631 *	5'-... *CGTTGCCAACCAGTA*CGGCCTTAA[TCCGT]GGACG...-3'
33986-34019	5'-...*CTGTTTAGTTACGAG*CGACATTGC[TCCGT]GTATT...-3'
34316-34283*	5'-...*TGTGATGGAACAATA*CCAGGACTA[TCCGT]ATGAC...-3'
43565-43598	5'-...*TCTTGCCCATAAAGC*AGATGAACT[TCCGT]TAATC...-3'
44551-44518*	5'-...*TATCATGCCGTTAATA*TATGTTGCCA[TCCGT]GGCAA...-3'

Base numbering refers to the conventional orientation of the lambda genome. Bold, a terminal portion of *Tsp*GWI-cut restriction fragment, T4 DNA polymerase repaired and cloned into pTZ18u derivative; italic, uncloned bacteriophage lambda DNA sequence adjacent to cloned *Tsp*GWI-derived restriction fragment; box with horizontal arrow, *Tsp*GWI recognition sequence; vertical arrows, *Tsp*GWI cleavage positions.
*Sequence obtained from *Tsp*GWI restriction fragments cloned in reverse complement orientation.

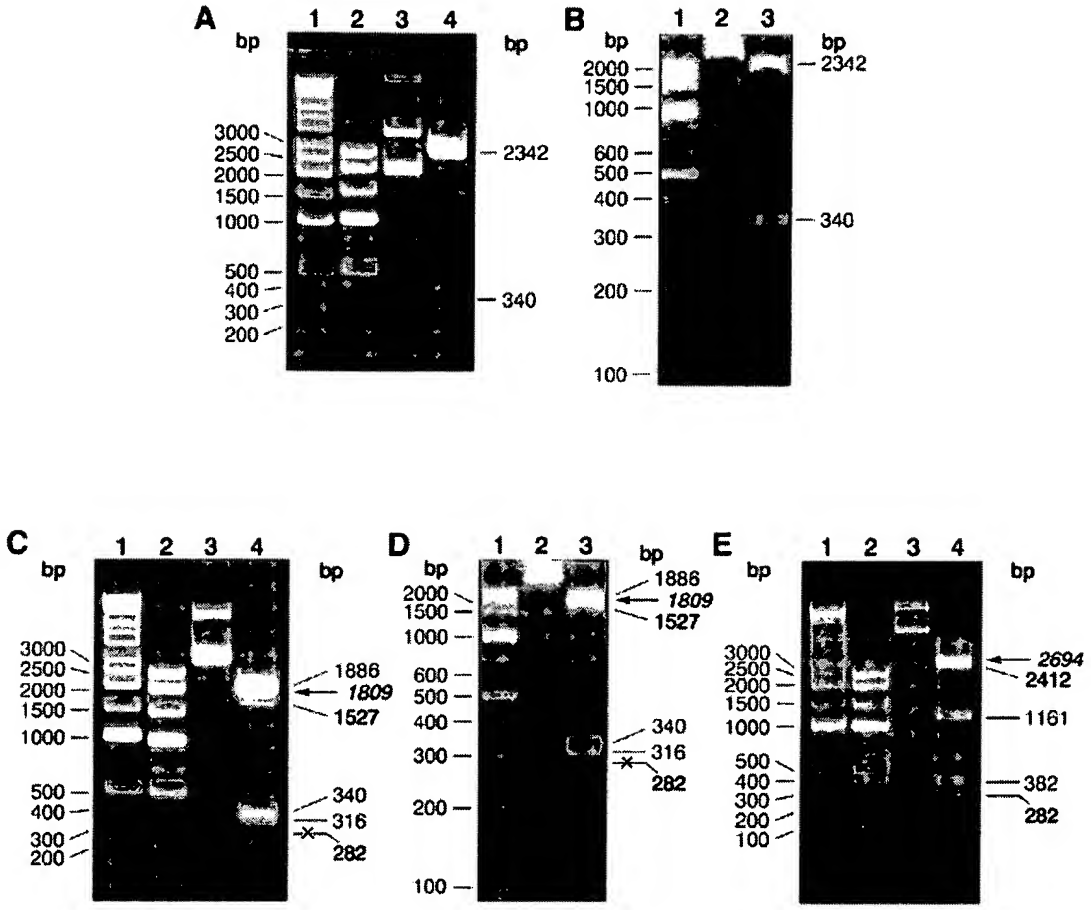
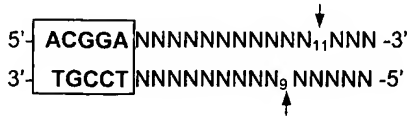


Figure 1. Digestion pattern and site preference of *Tsp*GWI on pUC19, pBR322 and pACYC184 plasmid DNAs. (**A**) *Tsp*GWI cleavage of pUC19 DNA, 1% agarose/TAE. Lane 1, 1 kb ladder; lane 2, 100 bp ladder; lane 3, untreated pUC19 DNA; lane 4, *Tsp*GWI-cut pUC19 DNA. (**B**) *Tsp*GWI cleavage of pUC19 DNA, 3.5% NuSieve GTG agarose/TAE. Lane 1, 100 bp ladder; lane 2, untreated pUC19 DNA; lane 3, *Tsp*GWI-cut pUC19 DNA. (**C**) cleavage of pBR322 DNA, 1% agarose/TAE. Lane 1, 1 kb ladder; lane 2, 100 bp ladder; lane 3, untreated pBR322 DNA; lane 4, *Tsp*GWI-cut pBR322 DNA. Out of five *Tsp*GWI sites in pBR322, one 5′-AACGGAT-3′ variant is cleaved inefficiently, resulting in fragments of 1527 bp (faint band, marked in bold) and 282 bp (not visible on reproduced picture, marked in bold and crossed). The partial digestion band of 1809 is indicated by bold italics and a horizontal arrow. (**D**) *Tsp*GWI cleavage of pBR322 DNA, 3.5% NuSieve GTG agarose/TAE. Lane 1, 100 bp ladder; lane 2, untreated pBR322 DNA; lane 3, *Tsp*GWI-cut pBR322 DNA. (**E**) *Tsp*GWI cleavage of pACYC184 DNA, 1% agarose/TAE. Lane 1, 1 kb ladder; lane 2, 100 bp ladder; lane 3, untreated pACYC184 DNA; lane 4, *Tsp*GWI-cut pACYC184 DNA. Bands of 2412 and 282 bp, where intensity is decreased due to slow cleavage rate of *Tsp*GWI site variant 5′-AACGGAT-3′, are marked in bold. Partial digestion band of 2694 bp is marked in bold italics and a horizontal arrow. Selected band sizes of marker DNAs are shown on the left of each panel.

orientation. Comparison of the junction sequences confirmed the presence of the 5′-ACGGA-3′ site at either defined distances near the ends of a cloned restriction fragment or in pBR322/lambda DNA regions which, prior to *Tsp*GWI cleavage, were continuous with their corresponding cloned fragment. The sequences presented in Table 1 were selected to show all found combinations of the neighboring 1 bp, flanking both sides of the *Tsp*GWI recognition sequences. The computer prediction revealed 107 5′-ACGGA-3′ sites in lambda. Four out of the 16 possible combinations of 1-bp neighborhoods are under-represented in the lambda genome and they were also not detected during the junction sequence analysis (marked in bold): 5′-AACGGAT-3′ (5/107), 5′-AACGGAC-3′ (6/107), 5′-TACGGAC-3′ (1/107) and 5′-TACGGAG-3′ (0/107). Since the 5′-AACGGAT-3′ site present in the tetracycline resistance gene is slowly cleaved this may apply to lambda as well. This would decrease the cloning efficiency of 5′-AACGGAT-3′ fragments. Whether the absence of 5′-AACGGAC-3′ and 5′-TACGGAC-3′ sequences amongst the analyzed junctions is also caused by diminished reaction efficiency remains to be determined.

The *Tsp*GWI recognition sites from the sequenced junctions appear in four possible configurations: as top or bottom strand in either an insert or an uncloned segment of adjacent pBR322/lambda DNA regions (Table 1). The comparison of various clone configurations allowed the deduction of the cleavage positions, which are located further downstream from the 5′-ACGGA-3′ sequence, at the 11 and the 9 nucleotide in the top and bottom strands (vertical arrows), respectively, leaving 2 nt 3′-overhangs:



The recognition sequence and cleavage points of *Tsp*GWI show similarities to those of the two known *Thermus*-derived restriction endonucleases of type-IIS: *Taq*II from *Thermus aquaticus*, GACCGA($N_{11/9}$)-3′ or CACCCA($N_{11/9}$)-3′ (5), and *Tth*111II from *Thermus thermophilus* 111, CAARCA($N_{11/9}$)-3′ (6). The conservation of cleavage positions can be observed, as well as of the first and the last A of the *Tsp*GWI recognition site. This may indicate a common evolutionary origin for all these endonucleases.

The optimum reaction conditions for *Tsp*GWI are 50 mM Tris–HCl pH 8.5 at 25°C, 10 mM $MgCl_2$, 10 mM DTT and 3 mM spermidine. The enzyme is active between 42 and 85°C, with a maximum at 65 and 75°C. The enzyme can be inactivated by 20 min incubation at 89°C.

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